(12) UK Patent Application (19) GB (11) 2 247 889 (13) A

(43) Date of A publication 18.03.1992

(21) Application No 9019946.4

(22) Date of filing 12.09.1990

(71) Applicant
Scientific Generics Limited

(incorporated in the United Kingdom)

King's Court, Kirkwood Road, Cambridge, CB4 2PF, United Kingdom

(72) Inventor
Christopher John Stanley

(74) Agent and/or Address for Service
W H Beck, Greener & Co
7 Stone Buildings, Lincoln's Inn, London, WC2A 3SZ, United Kingdom

(51) INT CL[5]
C07H 21/04, C12N 15/10, G01N 33/53

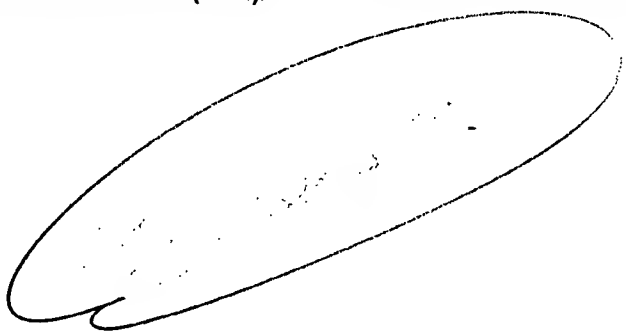
(52) UK CL (Edition K)
C3H HB4
U1S S1334 S1351

(56) Documents cited
Gen. Physiol. Biophys. 1985, 4, 219-237

(58) Field of search
UK CL (Edition K) C3H HB4 HB5
INT CL[5] C07H 21/04, C12N 15/10
Online databases: WPI, DIALOG/BIOTECH

(54) DNA denaturation by an electric potential

(57) A process is described for denaturing DNA into its individual strands in an electrochemical cell in the presence of a mediator compound that transfers charge from an electrode to the DNA in solution. The process may be used in the replication of DNA by a polymerase chain reaction (PCR).



GB 2 247 889 A

FIG. 1.

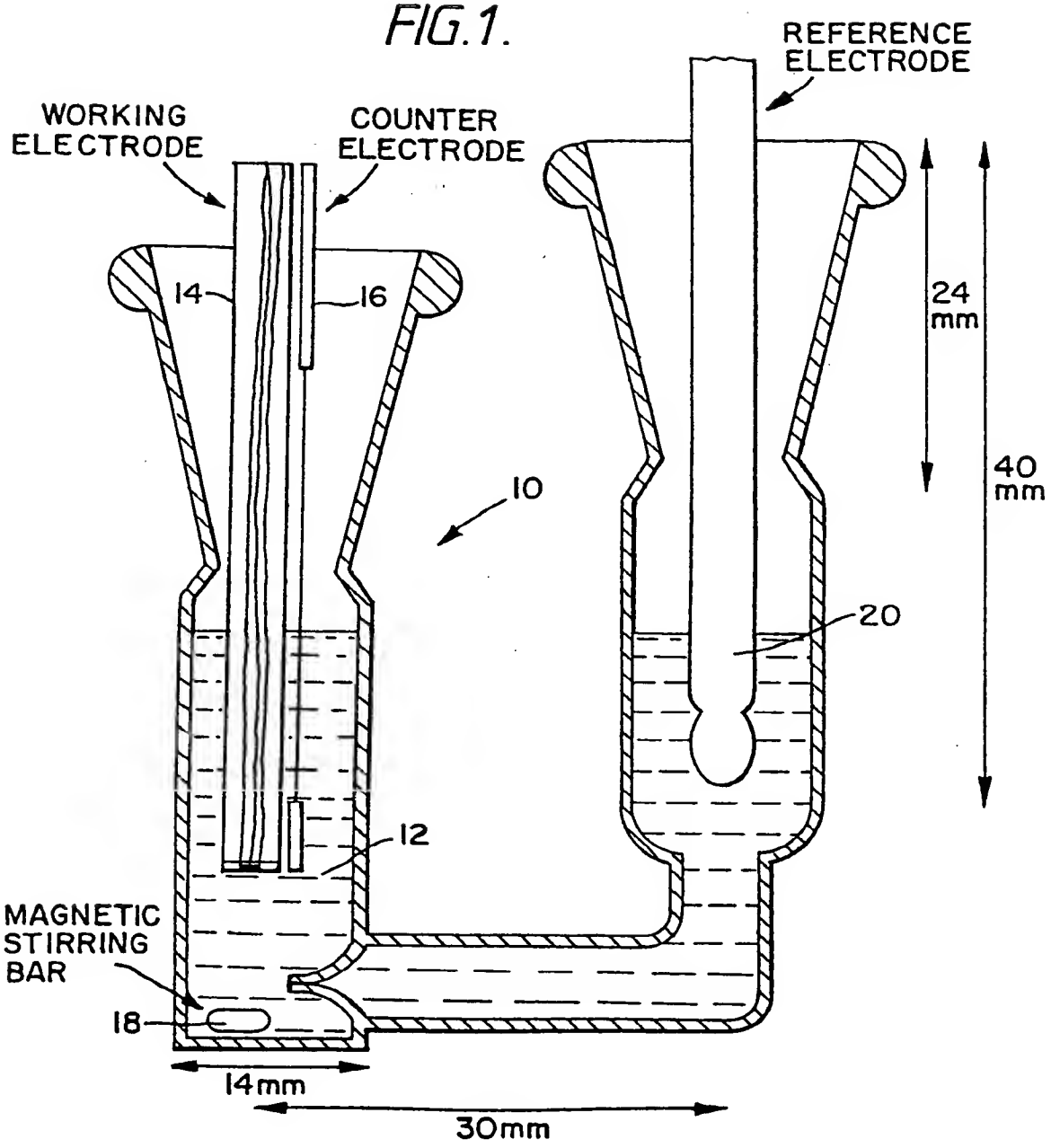
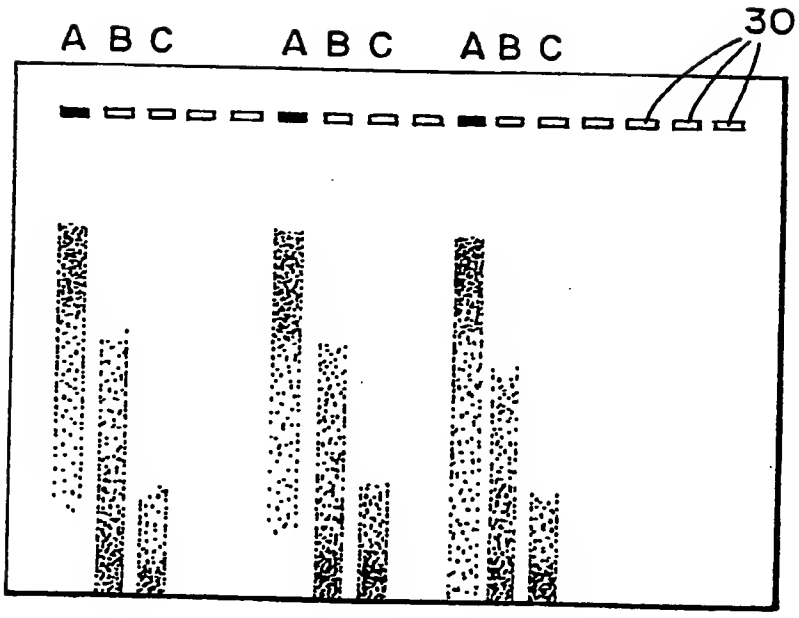

REFERENCE ELECTRODE
WORKING ELECTRODE
COUNTER ELECTRODE
14
16
24 mm
40 mm
10
20
12
MAGNETIC STIRRING BAR
18
14mm
30mm

FIG. 2.



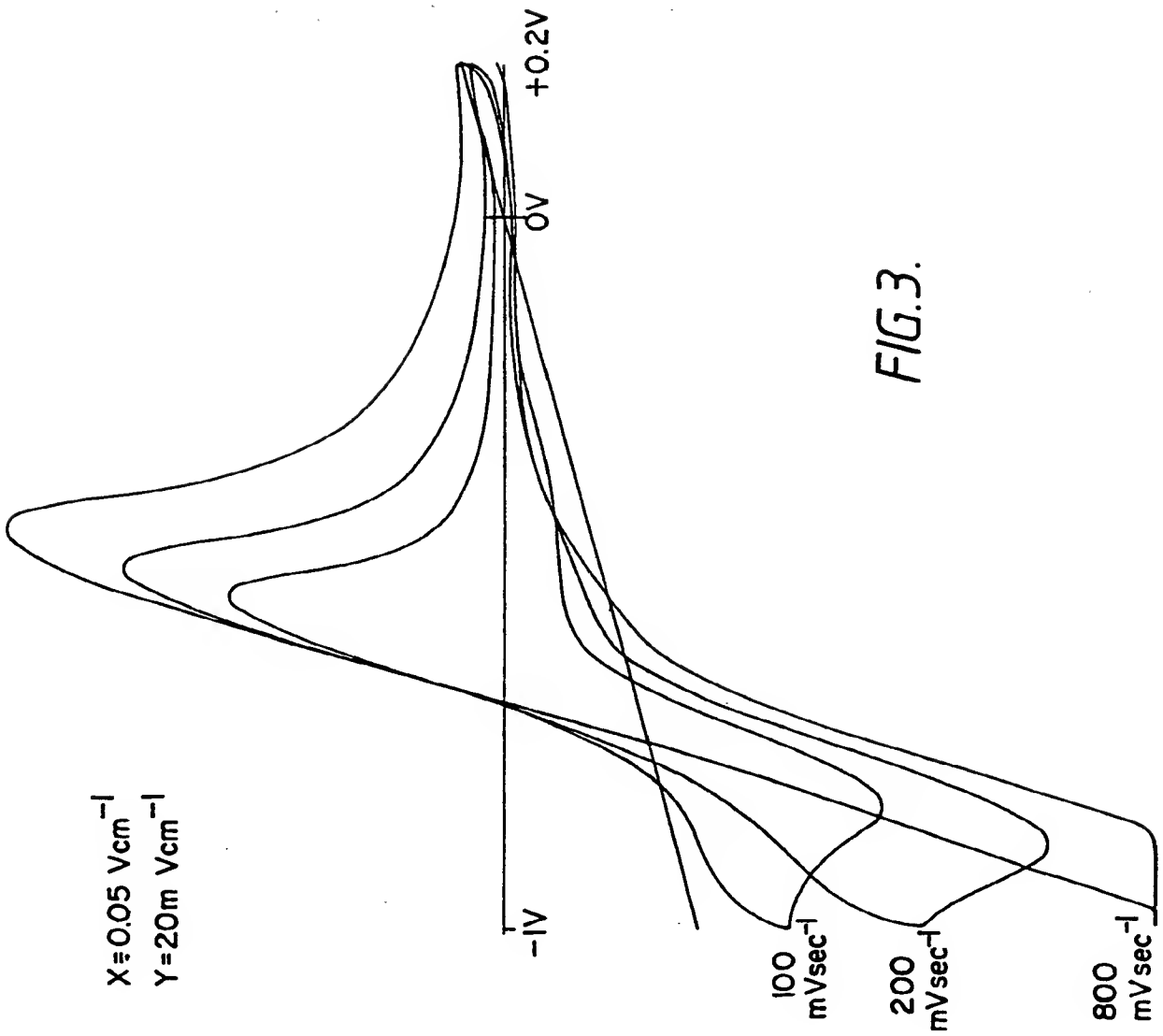

+0.2V
0V
-1V
X=0.05 Vcm⁻¹
Y=20m Vcm⁻¹
100 mVsec⁻¹
200 mVsec⁻¹
800 mVsec⁻¹

FIG.3.

TREATMENT OF NUCLEOTIDE MATERIAL

This invention relates to a process for the treatment of nucleotide material.

US Patent No 4683195 (Kary B Mullis et al, assigned to Cetus Corporation) discloses a process for amplifying and detecting a target nucleic acid sequence contained in a nucleic acid or mixture thereof by separating the complementary strands of the nucleic acid in the presence of oligonucleotide primers, extending the primers to form complementary primer extension products and then using those extension products for the synthesis of the desired nucleic acid sequence. The individual process steps can be carried out repetitively to generate large quantities of the nucleic acid sequence that may be required to be reproduced from even a single strand of the starting material. However, the step of separating the complementary strands of the nucleic acid is by thermal denaturation, and the need for thermal cycling limits the speed at which the multiplication process can be carried out, requires use of special heat resistant enzymes for the extension step, and can lead to evaporative loss of water.

It is therefore an object of the invention to provide a process for converting double stranded nucleic acids into single stranded nucleic acids which can be carried out at ambient or near ambient temperatures.

According to the invention double stranded nucleic acid may be converted into denatured single stranded nucleic acid in free solution by applying an electrical potential to a solution containing the nucleic acid.

H. W Nurnberg, "Applications of Advanced Voltammetric Methods in Electrochemistry" [H. W. Nurnberg et al (Editors), "Bioelectrochemistry", Plenum Inc (New York), 1983, pp. 183-225] discloses partial helix opening of adsorbed regions of native DNA to form a so-called ladder structure. However, the DNA is effectively bound to the electrode surface and separation of the individual strands to provide useful lengths of single stranded DNA in solution has not been demonstrated. The denatured single stranded DNA reported by Nurnberg either has reorientation of adsorbed bases or is chemically modified by reduction of adenin and cytosine in the single stranded regions.

F. Jelen and E. Paleck, "Nucleotide Sequence-Dependent Opening of Double-Stranded DNA at an Electrically Charged Surface", Gen. Physiol. Biophys., (1985), 4 , 219-237 discloses the opening of a DNA double helix on prolonged contact of the DNA molecules with the surface of a mercury electrode. The mechanism of opening of the helix is postulated to be anchoring of the polynucleotide chain via hydrophobic bases to the electrode surface after which the negatively charged phosphate residues of the DNA are strongly repelled from the electrode surface at an applied potential close to -1.2 volts, the strand separation being brought about as a result of the electric field adjacent to the electrode. There is no disclosure of separating the strands of the DNA double helix while the DNA is in solution rather than adsorbed onto the electrode, and there is no disclosure of single strand DNA in solution as a result of this treatment.

N. L. Palecek, "860-Electochemical Behaviour of Biological Macromolecules", Bioelectrochemistry and Bioenergetics, 15 , (1986), 275-295 discloses a peak in voltammogram measurements of DNA at a about -1.8 volts corresponding to chemical reduction of guanine to dihydroguanine, but this reduction does not

require opening of the double helix into its individual strands.

The process of the invention is believed, although the invention is not limited to this theory, to proceed by an electron transfer to the DNA while the DNA is in solution, the electron then weakening the hydrogen bonds between adenine and thymine or cytosine and guanine which can bring about the separation of the strands. The electron transfer is believed to be reversible, and there is no chemical modification of the DNA molecule itself or its individual strands, the strand separation or denaturing process being reversable and the strands being capable of re-hybridising in so far as re-hybridisation is not prevented by other unrelated factors of the DNA molecule itself or of the solution in which it is present. If, for example, the process is carried out at or adjacent to the pre-melting temperature of the DNA (i.e. the temperature at which the hydrogen bonds between adenine and thymine break and the cytosine-guanine bonds are ready to break but have not yet broken), the material may then not re-hybridize simply on removal of the voltage from the electrode.

Re-hybridisation will also, of course, not proceed if the temperature is too low.

The strand separation is brought about by an electron transfer process to DNA that is free in the solution rather than adjacent to and adsorbed onto an electrode that process preferably being the result of the presence in the solution containing the DNA of a mediator which receives electrons from the electrode and transfers them to the DNA to effect said strand separation. For the present purposes a mediator is defined to be an inorganic or organic molecule which is capable of reversible electron transfer at an electrode and which passes electrons on to or receives electrons from a biological molecule, in this instance the nucleic acid present in solution. Such mediators are described by M. L. Fultz et al, "Mediator compounds for the electrochemical study of biological redox systems: a compilation", Analytica Chemica Acta, 140 (1982), 1-18. The mediator should be soluble in the solvent for the DNA (which may be water or a solvent other than water) and compounds having a redox potential of 0 to -2 volts, preferably -0.2 to -1 volt and especially about -0.4 volts are preferred. Thus the mediator may be

a water or solvent soluble compound having conjugated or aromatic groups and one or more hetero-atoms and may be a compound of the quinone or bipyridyl series, especially a viologen such as methylviologen or a salt thereof. The choice of mediator is not believed to be critical provided that its redox potential is within the required voltage range and compound does not otherwise affect or interfere with DNA or other materials present in the solution such as enzymes or oligonucleotide probes. The use of a mediator enables the DNA or other nucleic acid material to be denatured into its individual strands at an applied voltage of -0.1 volt or less. Although denaturation has be n observed by the present inventors at a voltage of -1 volt, it is believed that this may be an overvoltage and the voltage needed to bring about actual denaturation may be as low as -0.8 volts especially since the redox potential of the mediator is typically 0.4 volts. The experiments reported herein have been carried out at negative electrode voltages, but the possibility of bringing about denaturation at positive electrode voltages by an electron transfer process in which electrons are removed from the DNA can not be excluded. In principle the application of an electric charge to

native DNA or similar nucleic acid material can bring about separation of the individual strands whether that charge is a positive or a negative electrical charge, the mechanism of separation being postulated to be the electrical repulsion of like charges injected or transferred into the nucleic acid material.

The process may be carried out in a cell in which there is a working electrode and a counter electrode. The working electrode may be of any convenient material e.g. a noble metal such as silver, gold or platinum, or it may be a carbon electrode. The electrode may be a so called "modified electrode" in which the electron transfer is e.g. by an electron donating or electron accepting compound such as a mediator coated onto, or adsorbed onto, the surface of the electrode which is otherwise of an inert material. The electron transfer may also be from or to an electrode consisting at least partially of a mediator compound e.g. formed wholly of the mediator compound. In a practical cell the working, counter and reference electrodes may be formed on a single surface e.g. a flat surface by e.g. thick film screen printing, ink jet printing, or by using a photo-resist followed by

etching. Electrode arrays may be provided for multiple DNA experiments.

The strand separation may be carried out in an aqueous medium or in a solvent other than water, for example a mixture of water with an organic solvent such as tetrahydrofuran, alcohol or acetone, or it may be carried out in a polar organic solvent e.g. acetonitrile. The use of non-polar solvents is not at present excluded, but is not preferred. The process may be carried out at temperatures less than 100°C e.g. ambient temperatures or if desired temperatures adjacent the pre-melting temperature of the DNA or other biologically active compound e.g. at temperatures of about 60°C. The process may be carried out pH's of from 3 to 10 conveniently about 5.5. The DNA may be dissolved in an aqueous solution containg a buffer whose nature and ionic strength are such as not to interfere with the strand separation of the DNA. The strand separation reaction may take up to 24 hours or more depending upon the nature of the DNA sample and the mediator employed. Strand separation is believed to have been observed in the absence of mediator but proceeds more slowly.

It is preferred that the process of strand separation should be carried out in a cell in the absence of stirring or under gentle stirring conditions. This not only assists in preventing shearing of the DNA but also is believed to facilitate electron transfer to the DNA with a low degree of entrapment of the DNA on the electrode surface. The reason is that the mediator being a much smaller molecule than the DNA diffuses rapidly through the solution compared to the DNA so that the transport of the reduced form of the mediator from the electrode surface to the DNA molecule is a rapid process compared with the diffusion of the DNA molecule towards the electrode. In a case of a negatively charged electrode, the mediator will transfer electrons to the DNA which will itself acquire a negative charge and therefore will tend not to reach the surface of the electrode but to be maintained in solution. It is therefore possible that the process could be carried out in a two compartment cell divided by a membrane, the DNA solution being in a compartment remote from the cathode, and the electrons being transferred from the cathode to the DNA by the mediator which the cell membrane is permeable.

The process of the invention is believed to be industrially applicable because the single strand DNA or biologically material is useful in its own right. For example, it may be used to provide a stock of single stranded DNA material for hybridisation studies during research and as a molecular weight marker for gel electrophoresis testing.

It may also be used for hybridisation studies with an indicator probe to identify a gene sequence e.g. specific to a particular organism or specific to a particular heredetary disease of which sickle cell anaemia is an example. Thus invention provides a process for detecting the presence or absence of a predefined nucleic acid sequence in DNA or other nucleotide material which comprises:

denaturing a sample of DNA under a voltage supplied to the sample DNA in solution by means of electrode;

hybridising the denatured DNA with an oligonucleotide probe for the sequence; and

determining whether the hybridisation has

occurred. The determination may be by means of a radio-labelled, optically labelled or enzyme-labelled probe.

The standard procedure for carrying out a DNA probe assay involves the preparation of double-stranded DNA from the sample, its denaturation by heat or alkali or both, the binding of the resulting single stranded material to a solid support e.g. a filter, addition of a labelled probe for the sequence of interest and hybridising at 25 to 45^{o}C, optionally in the presence of organic solvent such as dimethyl formamide, washing away excess labelled probe and detecting the label. This is a complex and time-consuming process. One improved method for carrying out DNA probes is the so called "sandwich" technique where a specific oligonucleotide is immobilised on a plastic surface. The surface having the specific oligonucleotide thereon is then hybridised with a solution containing the target DNA in a single-stranded form, after which a second labelled oligonucleotide is then added which also hybridises to the target DNA. The plastic surface is then washed to remove unbound labelled oligonucleotide, after which any label which has

become bound to target DNA on the surface can be detected later. This procedure can be simplified by using the present process to denature the double-stranded DNA into the required single-stranded DNA. The working electrode and mediator can be incorporated into a test tube or a well in which the DNA probe assay is to be carried out. The DNA sample and oligonucleotide primers can then be added, the reducing voltage applied to denature the DNA and then remove, the resulting single-stranded DNA is hybridised with the specific oligonucleotide immobilised on the plastic surface, after which the remaining stages of a sandwich assay are carried out. All the above steps can then take place without a need for high temperatures or addition of alkali reagents as in the conventional process.

For the so called "filter support" form of DNA probe assay, the target DNA may be bound to the filter which is then saturated with mediator, after which the filter may be physically applied to a working electrode (which may be a sheet or a dot array) after which a reducing voltage is applied to the electrode to denature the DNA in situ on the filter. Labelled oligonucleotide may then be added, after

which the filter is washed and any remaining labelled oligonucleotide is detected e.g. by fluorescence or by radio-active counting depending upon the nature of the label. It is also possible to apply just the appropriate voltage during hybridisation of the labelled probe to ensure binding thereof. In this way the requirement for baking of filters to achieve denaturation of DNA bound theron is avoided - in fact it may be possible to avoid taking the assay away from room temperature when electrochemical denaturation is used.

A further use of electrochemical denaturation of DNA probes is in histochemistry where labelled DNA probes can be hybridised in situ to tissue sections on a microscope slide. This procedure is currently carried out by heating a tissue slice on the slide to a high temperature in order to denature the target DNA. According to the invention the target DNA may be denatured electrochemically by soaking the tissue slice in a solution containing a mediator and applying a reducing voltage e.g. by using an electrode incorporated into or formed on the surface of the microscope slide itself.

The denaturing proc ss of th invention may also be

used in gel electrophoresis. A gel of agarose or polyacrylamide containing double-stranded DNA (which may have been separated by charge or size) cannot be heated or treated with alkali to denature the DNA into its single strand because the gel collapses. Instead the gel may be soaked with a mediator, placed on a working electrode (a flat plate or array) after which a reducing voltage is applied to denature the DNA in the gel. The DNA in the gel can then be subjected to electrophoresis a second time, preferably to give a two-dimensional gel in which the first electrophoresis dimension is carried out with double-stranded DNA and the second electrophoresis dimension is carried out with single-stranded DNA or with single-stranded DNA which has been hybridised with a labelled oligonucleotide probe to enable specific sequences to be detected. The possibility of carrying two-dimensional gel electrophoresis of DNA, the first dimension corresponding to double-stranded DNA and the orthogonal dimension corresponding to single-stranded DNA, could provide a new technique for DNA analysis permitting the detection of component single strands of unequal length in double-stranded pieces of DNA. The ability to denature the DNA within the gel and to hybridise the

resulting single-stranded DNA directly with labelled oligonucleotides is an advantage because it avoids the need to remove the appropriate portions of the gel and separate the double-stranded DNA therefrom before the denaturing and hybridisation can be carried out.

This process can be further expanded to achieve three-dimensional electrophoresis of DNA, that is movement in all three planes within the gel. An electrode can be used to draw probe sequences into a gel from the surface above the separated samples. This can be achieved because large DNA sequences will move very slowly through the gel towards the electrode, but small sequences move much more quickly and could overtake and hybridise with the larger sequences.

The invention also has application in cytology because fragile materials such as cells can be analysed for the presence of specific sequences in their DNA by making the cells permeable to mediator if that is not already the case, applying a reducing voltage to denature the genomic, plasmid, mitochondrial or chloroplast DNA, having a labelled oligonucleotide probe to the mediator permeable

cells and causing the probe to hybridise with the denatured DNA to enable particular sequences to be detected. Excess probe and mediator can be removed by dialysis and the cells can then be sorted and analysed by flow cytometry and/or by microscope examination. The fragile structures of the cell can be preserved during this method whereas the heat or alkali required for conventional methods of DNA denaturing would cause irreversable changes in the cellular appearance.

The invention may also be used as one step in the amplification of viral or DNA vectors. These vectors commonly exist in their native form as single-stranded DNA, and exist in the cell cultures in intracellular form as a double stranded DNA. Lysis of the cells to recover the viral DNA which has grown gives rise to the double stranded form of the viral DNA which has to be converted back into the single stranded native form of the virus. The process of the invention may be used to carry out that step.

The invention may further be used for gene replication in a process analogous to the existing polymerase chain reaction (PCR) for replicating DNA.

Thus in a further aspect the invention provides a process for replicating DNA which comprises:

separating the strands of a sample DNA in solution under the influence of an electrical voltage applied to the solution from an electrode;

hybridising the DNA with at least one oligonucleotide primer (see US Patent 4683195) that hybridises with at least one of the strands of the denatured DNA;

synthesising an extension product of the or each primer which is sufficiently complementary to the respective strand of the nucleic acid to hybridise therewith (see also US Patent 4683195); and

separating the strand of an extended DNA from the nucleic acid strand with which it is hybridised to obtain the extension product.

In the above process the hybridisation step may be carried out using a single primer that hybridises with only one of the strands, but for amplification it is desirable to use two primers which are complementary to the different strands of the DNA.

The denaturation to obtain the extension products can be carried out by applying to a solution of the extension product a voltage from the electrode. The process may be carried out stepwise as in the existing thermal method of PCR, but it may also be carried out continuously because as soon as the extension product is formed it can be broken down by the electrode voltage so that there is no need to wait for complete separation of the strands of the original sample before the separation of the extension products can begin. Thus, provided that the primer will hybridise with the DNA a denaturation product will grow, and the technique can be used analytically to determine in a very small sample the DNA e.g. a single strand in the presence of a particular nucleotide sequence which is then amplified into quantities sufficient for bulk analysis.

The invention will now be described with reference to the following drawings and examples.

Figure 1 is a diagram of a test cell;

Figure 2 is a drawing of a developed electrophoresis gel plate showing the movement of single and double stranded DNA, the forms of DNA being firstly a stock DNA, secondly an electrically denatured DNA according to the invention and thirdly a thermally denatured DNA;

Figure 3 is a voltammogram obtained using a solution of DNA electrically denatured into its individual strands according to the invention; and

The invention is illustrated by the following example.

Example

In Figure 1 there is shown a cell structure 10 comprising the working compartment 12 in which there is a body of DNA-containing solution, a working electrode 14, a counter electrode 16 and a magnetic stirrer 18. A reference electrode 20 in a separate side arm is connected via a "luggin" capillary to the solution in the sample 12. The working electrode, counter electrode and reference electrode are connected together in a potentiostat arrangement so that a constant voltage is maintained betwe n the

working electrode 14 and the reference electrode 20, the counter-electrode 16 controlled by an amplifier feedback loop to maintain thus set potential. Such potentiostat arrangements are well known.

1.6 of a solution in distilled water of methylviologen dichloride, the solution containing 1mg of that compound per ml was filled into working compartment of the electrochemcial cell. The reference arm of the cell containing the electrode 20 contained 0.4ml of this solution. A sample of 120 microlitres of a stock solution of calf thymus DNA (Sigma Chemical Company D4522, average molecular weight 5Kb) containing 1mg per ml was added to the working chamber and a voltage of -1.0 volts was applied between the working electrode 14 and the reference electrode 20. The cell was left overnight without stirring. A blue colour from reduced viologen was visible in the immediate vicinity of the working electrode 14. The cell was left unstirred because it was considered that the diffusion rate of the viologen would be much greater than that of the double stranded DNA so that the viologen might transfer electrons to the DNA without the DNA itself contracting the lectrode 14

and becoming bound thereto. This would reduce the possibility of irreversible coating of the electrode. A 100 microlitre sample of the material in the working part of the cell was taken at the end of this period and prepared for gel electrophoresis analysis by mixture with 20 microlitres of gel loading buffer which contained 0.25 per cent w/v of bromophenol blue, 0.25 per cent w/v xylene cyanol, and 30 per cent v/v of glycerol, the balance being water. The resulting DNA solutions were loaded in 10 microlitre wells formed in an electrophoresis gel. The gel (Figure 2) had a number of wells 30 into which the samples could be inserted, and nine 10 microlitre samples were placed into individual wells. The gel had a total volume of 30ml and was 10cm wide and 7.5cm long; it was 0.5 per cent w/v agarose in TBE buffer containing 0.089 M tris(hydroxymethyl)methyl ammonium borate and 0.01 M of EDTA, the gel having a pH of 8. The gels were incubated for 80 to 90 minutes at an applied voltage of 55 volts and then stained by addition of 0.75 ml of ethidium bromide (2.0 mg/ml). The stained gels were trans-illuminated with uv light and photographed using a red filter to reduce background from the uv sourc . The appearance of the resulting gel is shown in Figure 2.

Applied to each electrophoresis gel were three sets of samples A, B and C to give the nine test solutions referred to above. Sample A was the starting material used in the test with calf thymus DNA. Sample B was a sample of the calf thymus DNA which had been electrically denatured according to the invention and sample C was a sample of thermally denatured single stranded DNA. It will be noted that the samples B and C are of high mobility through the gel indicating that the DNA in the cell had been electrically denatured into single strands.

The above experiment was repeated as before, but samples were taken after 1.5 hours, 3 hours and 22 hours. A gradual progressive denaturation of the DNA into single strands was observed. After 1.5 hours there was a mixture of partially and fully denatured DNA but no evidence of wholly native DNA, and in later samples the proportion of fully denatured DNA increased upto 22 hours.

Figure 3 is a cyclic voltammogram of the material from the cell after 22 hours. The positive peak at -0.45 volts was attributed to the reduction of the

methyl viologen mediator and demonstrates that electron transfer is taking place.

CLAIMS

1. A process for converting double stranded DNA into single stranded DNA which comprises applying an electrical potential to a solution containing the DNA to separate the strands thereof.

2. A process according to claim 1, wherein the strand separate is brought about by an electron transfer process within the solution of the DNA.

3. A process according to claim 2, wherein the solution in which an electrode is present contains DNA together with a compound which receives electrons from the electrode and transfers them to the DNA to effect said strand separation.

4. A process according to claim 3, wherein the compound has a redox potential of 0 to -2 volts.

5. A process according to claim 4, wherein the compound has a redox potential of -0.2 to -1 volt.

6. A process according to any of claims 3 to 5, wherein the strand s paration is carried out using an aqueous solution of the DNA and compound, and

the compound is methyl viologen, benzyl viologen, neutral red or phenosafranin or a salt thereof.

7. A process according any preceding claim, wherein the electrode is of carbon, silver, gold or platinum.

8. A process according to claim 1 or 2, wherein the electron transfer is by an electron donating compound coated or adsorbed onto the surface of an otherwise inert electrode.

9. A process according to claim 1 or 2, wherein the electron transfer is from an electrode consisting at least partly of a compound that undergoes a redox reaction and which can transfer electrons to DNA as a result of said reaction.

10. A process according to any of claims 1 to 5, wherein the strand separation is carried in a solvent other than water.

11. A process according to any preceding claim, carried out at a temperature less than $100^{o}C$.

12. A process according to any preceding claim carried out at ambient temperatures.

13. A process according to any of claims 1 to 11, carried out at the pre-melting temperature of the DNA.

14. A process according to any preceding claim carried out at the pH of 3 to 10.

15. A process according to any of claims 1 to 13 carried out a pH of about 5.5.

16. A process according to any preceding claim, wherein the DNA is dissolved in an aqueous solution containing a buffer whose nature and ionic strength are such as not to interfere with strand separation of the DNA.

17. A process according to claim 16, wherein the DNA is dissolved in an aqueous solution whose ionic strength is 5mM or less.

18. A process for replicating DNA which comprises:

separating the strands of a sample DNA in solution under the influence of an electrical voltage applied to the solution from an electrode;

hybridizing the DNA with at least one oligonucleotide primer that hybridises with at least one of the strands of the denatured DNA;

synthesising an extension product of the or each primer which is sufficiently complementary to the respective strand of the nucleic acid to hybridize therewith; and

separating the strand or extended DNA from the nucleic acid strand with which it is hybridised to obtain the extension product.

19. A process according to claim 18, wherein the hybridization step is carried out using two primers which are complementary to different strands of the DNA.

20. A process according to claim 18 or 19, wherein the denaturation to obtain th extension products is carried out by applying to a solution of the extension product a voltage from an electrode.

21. A process for detecting the presence or absence of a predetermined nucleic acid sequence in DNA which comprises:

denaturing a sample of DNA under a voltage applied to the sample in solution by means of an electrode;

hybridizing the denatured DNA with an oligonucleotide probe for the sequence; and

determining whether the said hybridization has occurred.

Published 1992 at The Patent Office, Concept House, Cardiff Road, Newport, Gwent NP9 1RH. Further copies may be obtained from Sales Branch, Unit 6, Nine Mile Point, Cwmfelinfach, Cross Keys, Newport, NP1 7HZ. Printed by Multiplex techniques ltd, St Mary Cray, Kent.